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The interaction between XBP1 and eNOS contributes to endothelial cell migration

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Abstract：

The X-box binding protein 1 (XBP1) is a pivotal transcription factor in the endoplasmic reticulum stress response. Our previous studies have proven that XBP1 is involved in vascular endothelial growth factor (VEGF)-mediated endothelial cell (EC) proliferation and angiogenesis. In this study, we used EC monolayer wound healing, tube formation and transwell migration models to explorethe role of *XBP1*splicing in EC migration. We found that scratching on EC monolayer triggered *XBP1*splicing, which was attenuated by the presence of SU5416and LY294002, suggesting that VEGF signalling pathways may be involved. Over-expression of the spliced XBP1 (XBP1s) via Ad-*XBP1s*gene transfer increased while knockdown of *IRE1α*or *XBP1*by ShRNA lentivirus suppressed EC migration. Over-expression of XBP1s up-regulated the nitric oxide synthase 3 (*NOS3*)mRNA through the 3'UTR-mediated stabilization and increased eNOS protein translation. Further experiments demonstrated that miR-24 participated in the XBP1s-induced eNOSup-regulation and EC migration.Further co-IP and immunofluorescence staining assays revealed that protein kinase B (Akt), eNOS andXBP1s form a complex, resulting inAkt and eNOS nucleus relocation.These results suggest that *XBP1* splicing can regulate eNOSexpression and cellular location, leading to EC migration and therefore contributing to wound healing and angiogenesis.

Introduction

Cardiovascular diseaseis one of the leading causes of death worldwide. It is a multifactorial multi-steps disease, in which the endothelium injury is the initial step of the disease development. Endothelial cells(ECs) are key cellular components of the blood vessels, forming an intact mono-layered structure of vasculature that acts as a semi-permeable barrier between body fluids and the tissues, which modulating vessel tone via nitric oxide production[1, 2]. Endothelium injury is usually caused by risk factors or mechanical forces-induced ECs apoptosis, leading to local permeability change and a serial of inflammatory reactions. The adjacent EC proliferation and migration towards the denuded area plays an important role in vascular injury repairsand prevention of cardiovascular disease development.

Endothelial nitric oxide synthase (eNOS)enzyme, encoded by the *NOS3* gene, is one of the three isoforms that synthesize nitric oxide (NO). NO produced by eNOShave the ability topromote vascular relaxation,antioxidant and anti-thrombus, which is essentailtoregulating vascular tone, cellular proliferation, leukocyte adhesion, and platelet aggregation[3-5].As its attribution to NO production, eNOSplays an essential role in cardiovascular system protection[6].

MicroRNAs (or miRNAs) comprise a novel class of small, non-coding endogenous RNA molecules(about 22 nucleotides) that regulate gene expression by directing their target mRNAs for degradation or post-transcriptional regulation[7, 8]. MiR-24is conserved in various species, and belongs to miR-23~27~24 clusteron human chromosome 9 and 19. MiR-24 has been shown to participate in regulation of vascular ECs proliferation, apoptosis, migration, inflammation and differentiation[9-11].

The X-box binding protein 1 (XBP1) is a transcriptional factor containing a unique basic-region leucine zipper (bZIP) domain, which was originally identified as its capability of binding to the cis-acting X-box presented in the promoter regions of human major histocompatibility complex class II genes[12]. Accumulating evidence showed that XBP1, downstream of the inositol requiring enzyme 1 alpha (IRE1α), is a stress-inducible transcription factor that existson both invertebrate and vertebrate cells and is crucial for cell survival under stress conditions[13-15].In mammalian cells, XBP1 is a key signal transducer upon the endoplasmic reticulum (ER) stress response. XBP1 was reported to be associated with human disease such as metabolism disease, vascular disease and so on[16, 17].Although ER stress is reported to be participated in vascular diseases[18-22], the role of XBP1 in vascular disease and its relationshipto NO have not been well understood. In the current study, we demonstrated that EC monolayer injury via scratching induces *XBP1* splicing, which in turn up-regulates and interacts with eNOS, leading to eNOS nuclear translocation that promotes EC migration.

Results

1. Wound in EC monolayer induced *XBP1* splicing, whichwas involved in EC migration.

XBP1 exists as unspliced (XBP1u) and spliced (XBP1s) forms via IRE1α-mediated unconventional splicing[15, 23]. The*XBP1* splicing was shown to be involved in EC

proliferation and apoptosis[24, 25]. To test whether *XBP1* splicing is also involved in ECs migration, an endothelium damage model was mimicked *in vitro* by scratching a confluent human umbilical vein EC (HUVEC) monolayer, followed by the observation on cell migration and the assessment onXBP1s protein expression at 6h and 24h post-scratching. As shown is Figure 1A, the scratching activated *XBP1* splicing as revealed by Western blot analysis. In order to verify whether the vascular endothelial growth factor (VEGF) signaling pathway was involved in the scratching-induced *XBP1* splicing, selective inhibitors(SU1498, PD98059 or LY294002)were included.The quantitative RT-PCR with specific primer sets for *XBP1u* and *XBP1s* revealed that scratching decreased *XBP1u* but increased *XBP1s* mRNA levels, which was abolished by the inhibitors (Supplement 1). These results suggest that endothelium damage activates *XBP1* splicing in a VEGF signalling pathway dependent manner.

The endothelium damage normally triggers the adjacent ECs migration to the injured area. To assess whether the scratching-induced *XBP1* mRNA splicing contributes to EC migration, the gain-of-function (via Adenoviral transfer of XBP1s) and loss-of-function (via shRNA lentivirus-mediated knockdown of *XBP1* or *IRE1α*) assays were performed in HUVEC scratching model. As shown in Figure 1B, the over-expression of XBP1s enhanced EC migrationobserved at 8h post scratching.The effect of Ad-XBP1s is shown in Supplement 1 B.As expected, the knockdown ofeither *XBP1* or *IRE1α*attenuatedthe wound healing(Figure 1C&1D). Further experiments with the tube formation on Matrigel assaysrevealed that knockdown of *XBP1* or *IRE1α*suppressed HUVECsbasal and VEGF-induced tube formation (Figure 1E). These results suggest*XBP1* splicing indeed contributes to EC migration.

2. Wound in EC monolayer up-regulated eNOS expression in an *XBP1* splicing-dependent manner.

Previous studies have shown that NO is a potent inducer of ER stress[26, 27], and eNOShas been shown to be involved in angiogenesis by supporting ECsmigration[28].Thus, we wondered whether there was a crosstalk between *XBP1* splicing and eNOS in scratching-induced wound healing. The eNOS protein levels and NO production were firstly detected following scratching of a confluent HUVEC monolayer. As shown in Figure 2A, eNOS protein level was dramatically increased at 6h post-injury and then gradually returned to the basal level as the incubation time proceeded. As expected, NO production ofthe culture medium was increased by scratching (Figure 2B). However, there is a time delay between

NO production andeNOS protein increase (Figure 2A versus Figure 2B). Further experiments revealed that the eNOS protein level increase might be due to *NOS3* mRNA increase, which was VEGF signalling dependent (Figure 2C).We chose 3 different VEGF signalling pathway inhibitors.SU1498, is a selective inhibitor of the VEGFR2, having negligible activity at several other serine/threonine and tyrosine kinases[29].PD98059 is a potent and selective inhibitor of MAP kinase kinases (MAPKK), MEK1 and MEK2 by binding to the inactive form of MAPKK and prevents activation by upstream activators, whichcan be used to study the role of MAPKK signalling[30]LY294002 is a potent, cell permeable inhibitor of phosphatidylinositol 3-kinase (PI3K) that acts on the ATP binding site of the enzyme[31].

As described above, both *XBP1* splicing and *NOS3* expression were up-regulated by the scratching, there might be direct link between these two events. To test this, the *XBP1* expression and splicing were down-regulated via shRNA-mediated *XBP1* and *IRE1α*knockdown respectively prior to the scratching experiments. As shown in Figure 2D and 2E, the scratching-induced up-regulation of *NOS3* mRNA (Figure 2D) and eNOS protein (Figure 2E) was abolished by knockdown of either *XBP1* or *IRE1α*. Further experiments with a tetracycline/doxycycline inducible system (Ad-*TRE-XBP1s* plus Ad-*Tet-on*) revealed that over-expression of XBP1s in HUVECs could increase eNOS protein level (Figure 2F) and NO production (Figure 2G). These results suggest that XBP1 splicing is sufficient and essential for endothelium damage-induced eNOSupregulation.

3. XBP1s increased *NOS3* mRNA stability via down-regulating microRNA *miR-24*.

The upregulation of an mRNA molecule can be derived from an increase of its transcription or stability or both. As XBP1s is an intact transcription factor, the up-regulation of eNOS by XBP1s may be due to transcriptional regulation. To test this, the effect of XBP1s on the *NOS3* promoter reporter expression (pGL3-*NOS3*-Luc reporter) was assessed. Surprisingly, overexpression of XBP1s significantly decreased the reporter geneexpression (Figure 3A). XBP1uand mature activating transcription factor 6 (ATF6N), another ER stresstransducer (28)exerted a slightlyinhibitory effecton *NOS3*promoter reporter expression(Figure 3A).These results imply that *XBP1* splicing may increase *NOS3* mRNA stability. It has been established that the 3' untranslated region (3'*UTR*) plays an important role in mRNA stability. To test whether *XBP1* splicing stabilises *NOS3* mRNA through its 3'*UTR*, the *NOS3* mRNA 3'*UTR* was cloned into pSiCHECK2 reporter system. There are different *NOS3* transcript variants with two types of 3'*UTR*, for which the transcript variants

tv1 and tv4 are the representatives. Both 3'*UTRs* (*TV1-3'UTR* and *TV4-3'UTR*) were cloned and their response to XBP1s was assessed in HUVECs. As shown in Figure 3B, over-expression of XBP1s significantly increased the reporter gene expression, indicating that XBP1s can increase *NOS3* mRNA stability.

microRNAs (miRNAs) are a group of short non-coding RNAs, capable of regulating mRNA stability via binding to mRNA 3'*UTR*. Through electronic analysis of the potential miRNA target sequences with the RNAwalk software, we found that*hsa-miR-24*, *hsa-miR-125*and*hsa-miR-214* possessed potential binding elements in both *TV1*- and *TV4*- 3'*UTR*. Quantitative RT-PCR analysis of these miRNAs following scratching revealed that all three miRNAs were dramatically down-regulated (Figure 3C). To test whether the down-regulation of these miRNAs was due to transcriptional suppression or increased degradation, exogenous miRNAmimics were introduced into HUVECs, followed by scratching and assessment of the miRNA levels. Compared to endogenous miRNAs, the transfection of exogenous miRNA mimics induced a huge accumulation of the miRNAs (Figure 3D). Interestingly, scratching of the ECs significantly decreased amount of exogenous *miR-125* and *miR-214*, while had no effect on exogenous *miR-24* (Figure 3D).Further experiments revealed that over-expression of XBP1s significantly decreased *miR-24* level but had no effect on the other two miRNAs (Figure 3E). These results suggest that there are different mechanisms involved in the regulation of *miR-24* and the other two miRNAs.

As all three miRNAs have potential binding elements in *NOS3* 3'*UTR* and are down-regulated by scratching, it is possible that their down-regulation contributes to *NOS3* stabilisation. To test this, exogenous miRNA mimics were introduced into ECs and subjected to scratching, followed by *NOS3* mRNA analysis. As shown in Figure 3F, only the exogenous *miR-24* mimics (premiR24) abolished scratching-induced *NOS3* up-regulation, while the other two miRNAs had no effect. Similarly, only the exogenous *miR-24* mimics but not the other two ablated XBP1s-induced eNOS protein (Figure 3G). These results suggest that the XBP1s-mediated down-regulation of *miR-24* contributes to *NOS3* mRNA and eNOS protein up-regulation.

4. miR-24 attenuated XBP1s-induced EC migration

To further explore whether the down-regulation of *miR-24*contributesECs migration,*miR-24* mimics (premiR24) were introduced into HUVECs and infected with Ad-*XBP1s* virus to over-express XBP1s, followed by EC migration assays with transwell and

wound healing models. As shown in Figure 4A and 4B, the XBP1s-induced EC migration was significantly attenuated by exogenous *miR-24*, suggesting that the down-regulation of *miR-24* contributes to XBP1s-induced EC migration.

5.eNOS was involved in EC migration via forming a complex with AKT and XBP1s.

It has been reported that the eNOS/NO/Akt signalling pathway contributes to endothelium damage-induced EC migration[32, 33]. As described above, endothelium damage up-regulates eNOS and NO production via XBP1ssplicing. Our previous study has demonstrated that XBP1s can activate Akt phosphorylation[25]. Thus, we wonder whether NO production and Akt activation contribute to XBP1s-induced EC migration. To test this, the eNOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME)[34], PI3K inhibitor LY294002 and PKG inhibitor KT5823were used to inhibit NO production and Akt activation, respectively. As shown in Figure 5A and supplement 2, LY294002 but not L-NAMEor KT5823abolished XBP1s-induced EC migration,suggesting that NO productionis not involved in XBP1s-induced EC migration. However, knockdown of *NOS3* by siRNA fully attenuated XBP1s-induced EC migration (Figure 5B). As L-NAME is eNOS function(producing NO) inhibitor, not eNOS protein inhibitor, these results suggested that eNOS protein is essential for XBP1s-induced EC migration.Immunoprecipitation assay pulled down XBP1s,Akt and eNOS togetherin XBP1s over-expressed cell (Figure 5C),indicating that these three proteins may form a complex. In order to identify the complex formation, VEGF stimulation was introduced and completed immunoprecipitation assay pulled down XBP1s, Akt and eNOS together(Supplement 3A).Furthermore,immunofluorescent staining revealed that Akt and eNOSwere relocated into the nucleus in the Ad-*XBP1s* infected cells(Fig. 5D), and the similar trend are detected in scratching and VEGF stimulated HUVECs(Supplement3B and 3C).These results suggest that XBP1s, Aktand eNOS may form a complex in the nucleus.

Discussion

Endothelium damage is the initial step of the development of multiple cardiovascular diseases. The adjacent EC migration plays a key role in repairing the damaged endothelium. However, the underlying mechanisms are still unclear. In this study, we demonstrated that endothelium damage could activate XBP1 splicing in a VEGF signalling dependent manner, and that XBP1s not only regulated eNOS expression but also formed a complex with eNOS and Akt, which may contribute to endothelium damage-induced EC migration.

Endothelium damage *in vivo* usually induces platelet aggregation releasingplatelet-derived growth factors and activates adjacent ECs secreting growth factors to increase the adjacent EC proliferation and migration in a paracrine and/or autocrine manner[35-38]. During this process, VEGF may play a vital role. Endogenous VEGF expression and its autocrine action could be regulated by other growth factors[39]. Our previous studieshave demonstrated that VEGF can activate XBP1 splicing through VEGF receptor 2, the kinase insert domain receptor(KDR) interaction with IRE1α[25]. In this study, we found that the wound-induced *XBP1* splicing in HUVECs was blocked by KDR antagonist SU5416, suggesting that an active KDR signalling is essential. Several signalling pathways can be activated following KDR activation, of which the PI3K/Akt and MEK pathways are two serine/threonine kinase pathways[40]. However, the participation of these two signalling pathways in *XBP1* splicing seems different in wound healing and VEGF stimulation. In this study, these two signalling pathways seemed involved in scratching-induced *XBP1* splicing as the *XBP1* splicing was blocked by either PD98059 (MEK inhibitor) or LY294002 (PI3K/Akt inhibitor). However, both inhibitors had no effect on VEGF-induced XBP1 splicing[25]. The discrepancy may reflect the fact that manygrowth factors can be secreted under scratching and there may be interaction among them. IRE1α is normally activated by autophosphorylation at Ser/Thr sites[41]. The involvement of the Ser/Thr kinase MEK or PI3K/Aktin *XBP1* splicing suggests that these kinases may directly activate IRE1α phosphorylation, which needs detailed investigation.

The eNOS expression andNO production play an essential role in the maintenance of vascular homeostasis[4, 42, 43].Dysfunction in eNOS activity may lead to endothelial dysfunction, which is associated with cardiovascular diseases such as hypertension and atherosclerosis[44].It has been reported that there is link between ER stress and NO production[45, 46].However, the direct crosstalk between eNOS and *XBP1* splicing remains unknown. In this study, we demonstrated for the first time that XBP1s not only regulates *NOS3* mRNA andeNOS protein expression and NO production but also physically interacts with eNOS. XBP1s increased *NOS3* mRNA stability through down-regulating *miR-24*, which has a binding element in *NOS3* mRNA 3'*UTR* and directs *NOS3* mRNA degradation. The increased stability of *NOS3* mRNA may activate a feedback transcriptional suppression of *NOS3* gene, in which XBP1s is involved.

eNOS protein is an enzyme with multiple functions. Coupling with co-factors, eNOS catalyses L-Arginine to produce NO, while under uncoupling conditions, eNOS produces

reactive oxide species[47, 48]. eNOS can also serve as a co-activator regulating gene transcription[49, 50]. In this study, we found that scratching of EC monolayer increased eNOS protein and NO production in an *XBP1* splicing dependent manner, and that over-expression of XBP1s increased eNOS protein and NO production. The delay of NO production following scratching-induced EC migration indicates that NO may play a less important role in EC migration, especially in XBP1s-induced EC migration. Indeed, the suppression of NO production via L-NAME had no effect on XBP1s-induced EC migration. Very importantly, in this study we found that XBP1s physically interacted with eNOS protein and Akt in the nucleus, suggesting that eNOS may act as co-activator or co-repressor of XBP1s and participate in gene transcriptional regulation. Further detailed investigation on identifying the target genes of the XBP1s/eNOS/Akt complex will surely extend the functions of both eNOS and XBP1.

In summary, endothelium damage triggers the paracrine/autocrine secretion of growth factors from the adjacent ECs, which may include VEGF. The activation of VEGF receptor KDR and the downstream MEK and PI3K/Akt pathways contribute to IRE1α-mediated XBP1 splicing. The XBP1s down-regulates *miR-24* transcription, which increases *NOS3* mRNA stability. The elevated *NOS3* mRNA may drive XBP1s to supress *NOS3* transcription via a feedback way on one hand, and increases eNOS protein level and NO production on the other hand. eNOS protein forms a complex with XBP1s and Akt, directing gene transcriptional regulation. The overall is to increase adjacent EC migration to repair damaged endothelium (Figure 6). This study adds new evidence on the role of *XBP1* splicing in angiogenesis through up-regulating EC proliferation and migration.

Experimental procedures

Materials

All cell culture serum and media were purchased from Gibico,while cell culture supplements were purchased from Sigma. Theantibody against Flag (ab190059) wasfrom Abcam. The antibodies against XBP1 (M-186,sc-7160), IRE1α (sc-20790),eNOS（sc-376542）, Akt1/2/3 (sc-81434) and GAPDH (sc-25778) were fromSanta CruZ Biotech. All secondary antibodies werefrom Dakocytomation. All microRNA reagents were purchased from Thermo Fisher Scientific. SU1498, L-NAME, LY294002, KT-5823, DMSO, DAPI, crystal violet and Nitrite/Nitrate Assay Kit (23479)were purchased from Sigma.

Cell Culture

Human umbilical vein ECs (HUVECs, ATCC-PCS-100-010) were cultured on 0.04% gelatin-coated flasks in M199 medium supplemented with 1ng/ml β-EC growth factor, 3μg/ml EC Growth Supplement from bovine neural tissue, 10u/ml heparin, 1.25μg/ml thymidine, 10% FBS, 100u/ml penicillin and streptomycin in humidified incubator supplemented with 5% CO_2. The cells were split every three daysat a ratio of 1:5.

Adenoviral and shRNA lentiviral infection

For adenoviral infection, HUVECs were incubated withAd-null, or Ad-*XBP1u* (unspliced XBP1) or Ad-*XBP1s* (spliced XBP1) virus at 10 multiplicity of infection (MOI) for 6h, and then culturedin fresh complete growth medium for time duration indicated in figure legends. For the tetracycline inducible system, HUVECs were co-infected with Ad-*TRE-XBP1s* (10MOI) and Ad-*Tet-on* (2MOI) viruses for 6h and cultured for the time duration as indicated in figure legends. On the induction of XBP1s expression, 1μg/ml doxycycline was added into the culture medium and incubated for time duration as indicated in the figure legends. For shRNA lentiviral infection,HUVECs were incubated with 100 transfection unit/cell of non-target shRNA or *XBP1 shRNA* or *IRE1α shRNA*lentivirus in the presence of 10mg/mL polybrene for 16h, followed by culture in fresh complete growth mediumfor 72h and subjected to further treatments.

Wound healing migration assay.

Wound was created by scratching confluent HUVECs in 6-well plates with 1ml tip.After removal of the cell debris and medium, fresh M199 medium containing 0.5% FBS was added and incubated for 6 hr. For the observation of cell migration, threescratching lines were created in each of three wells. Images were taken with at three different sites on each scratching line at 0 hr and 6 hr. The average migrated cells werecalculated from 3 sites/line x 3 lines/well x3 wells. For the assessments on RNA or protein samples, 10 (vertical) x 10 (horizontal) scratching lines were introduced into confluent HUVECs in ϕ100mm dishes.

Trans-well migration assay

The transfected and/or infected HUVECs were detached by using trypsin and resuspended in M199containing 0.5% FBS. A 100μl of 1×10^5 cells/ml cell suspension was added into the insert and 600 μ l of M199containing 0.5% FBS was added into the holder of the trans-well (8μm pore size), followed by incubation for 6 hr. The cells were then fixed with 4% paraformaldehyde (Sigma) for 15 minutes, and stained with crystal violet solution for 15 minutes. After removal of the cells inside theinsert, the migrated cells were observed under Nikon Eclipse TS100 microscope and images were taken byNikon Digital Sight system and processed with Adobe Photoshop software. Cells were calculated from 10 view/well x 3 wells using 10x or 20x lenses.

Tube formation assay

The tube formation assay was performed asdescribed previously[51]. Briefly, 100μl/well of growth factor-reduced Matrigel solution (Millipore) was added to8-well chamber plate and solidified at 37 °C. 1×10^5shRNA lentivirus-infected HUVECs (72h) were resuspended in 100ul M199 medium supplemented

with 5μg/ml VEGF or 0.1% BSA and added tomatrigel containing wells in triplicate and incubated in 37 °C humidified incubator supplemented with 5% $CO_2$for 6h.Tube formation was observed under Nikon Eclipse TS100 microscope and images were taken byNikon Digital Sight system and processed with Adobe Photoshop software. Tube numbers were calculated from 10 view/well x 3 wells using 10x lenses.

NO detection

Scratching(3x3) was introduced into confluent HUVEC monolayer in 6-well plates. After removal of the cell debris, fresh M199 medium was added and incubated for time duration as indicated in the figure legends. The cell medium was collected and spun at 2000xg at 4ºCfor 5 minutes to remove cell debris. Eighty μl/wellof supernatant were added to 96-well platein triplicate and subjected to total NO [NO3- + NO2-] detection with Nitrite/Nitrate Assay Kit (Sigma) according to the protocol provided.

MicroRNA analysis

The cellular total RNA was isolated with mirVana™ miRNA isolation kit accordingto the manufacturer's instruction. The reverse transcription of miRNAs was performed with the AppliedBiosystems® TaqMan® MicroRNA Reverse Transcription kit and Applied Biosystems® 5x RT primer. The quantitativePCR amplifications of samples were done via Applied Biosystems® TaqMan® Universal PCR Master Mix,No AmpErase® UNG and 20x TaqMan small RNA Assay with protocol provided.

Pre-mir RNAs and siRNA transfection

For transienttransfection assay, HUVECs were seeded in 6-well plates at 5×10^4 cells/well in triplicate 24h prior totransfection and treated with serum and antibiotics free M199 medium 1h prior to transfection. Twentypmolof pre-miRRNAs were transfected into HUVECswith 9μl of lipofectamine RNAiMax (Thermo Fisher Scientific) according to the procedure provided. Six hourslater, the transfection solution was removed and fresh complete growth medium was added and the cells wereincubated and subjected to further treatment as indicated in the figure legends. For siRNA transfection assay, HUVECs were seeded in 25ml flasks 24h prior to transfection and treated with serum and antibiotics free M199 medium for 1h prior to transfection. Tenμl of 10μmol/L controlsiRNA or *NOS3 siRNA* (sc-36093)was transfected into HUVECs using 12μl of LipofectamineRNAimax (Invitrogen) according to the protocol provided. Fresh complete growth medium was added 6h post transfection and incubated for 48h. The cells were infected withAd-null orAd-XBP1s virus at 10MOIand incubatedfor 24 h, followed by trans-well migration assay.

Luciferase Activity Assay

HUVECs were seeded in 12-well plate at $5x10^4$cells/well 24h prior totransfection. HUVECs were transfected with (1) 0.1μg/well of pGL3-*NOS3-Luc* reporter and 0.02μg/wellof Renilla-Luc (internal control) together with 0.1μg/well of pShuttle2-*XBP1s*, pShuttle2-*XBP1u* or pShuttle2-*ATF6N* expression plasmids or (2) 0.1μg/well of psiCHECK2-*TV1-3UTR* or psiCHECK2-*TV4-3UTR*reportertogether with 0.1μg/well of pShuttle2-*XBP1s*and incubated for 36h, followed by luciferase activity assay.pShuttle2

plasmid was included as vector control (Mock). The relative luciferase activity (RLA) was defined as the ratio of the reporter luciferase activity to the internal control luciferase activity with that of mock group set as 1.0.

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed according to standard procedures described elsewhere. Briefly, the cells were lysed with IP-A buffer [10 mM Tris-HCl, pH7.5, 120 mMNaCl, 1 mML EDTA,1% Triton X-100 plus protease inhibitor cocktail tablets (one tablet for 50 mL, Roche)] and the protein concentrationwas measured by BioRad protein assay (BioRad)). One milligram lysate was mixed with 10µl of anti-FLAG-agarose beads (Sigma) and 3 volumes of IP-B buffer (IP-A buffer without Triton X-100) and incubated on a rotator at 4ºC for 4h, followed by SDS-PAGE and standard Western blot analysis. For the input control or routine Western blot, SDS-denatured 50µg lysate was applied to SDS-PAGE. After transferring to PVDF membrane(Amersham), the target protein bands were detected with primary and secondary antibodies incubation, ECL development and X-ray filmexposure (Amersham). The images were processed by Adobe Photoshop software.

RT-PCR and qPCR

The total cellular RNA was extracted using Qiagen RNeasy Kit according to theprotocol provided. Two microgram RNA was transcribed into cDNA using Improm-IIreverse transcription system (Promega). For realtime PCR (qPCR), 20ng cDNA was amplified by a real time PCRSYBR master mix (Applied Biosystems). Primers for qPCR were designed with thePrimer Express Software (Applied Biosystems) as follows:*XBP1u*(forward) 5′-tgctgagtccgcagcactag-3′, *XBP1s* (forward) 5′-tgctgagtccgcagcaggtg-3′anda common reverse primer 5′-gctggcaggctctggggaag-3′;*NOS35′*- aggaacctgtgtgaccctca -3′and 5′ -cgaggtggtccgggtatcc -3′; β-actin 5’-cacaactgggacgacatggag-3’ and 5’-ttcatgaggtagtcagtctgg-3’ as internal control.

Immunofluorescence staining

The cells on slides were fixed with 4% paraformaldehyde at room temperature (RT) for 15min and permeabilised with 0.1% Triton X-100/PBS at RTfor 15min, followed by blocking with diluted normal serum (1:20) for 1h, incubationwith diluted primary and secondary antibodies at 37ºC for 1h and 45min, respectively. Nucleus was counterstained with DAPI. Images were taken by using SP5 confocalmicroscope (Leica, Germany) and processed by Adobe Photoshop software.

Statistical Analysis

Data expressed as the mean ± S.E. were analyzed using GraphPad Prism software (version 6) with t test for pair-wise comparisons or analysis of variance, when t test was inappropriate, followed by Dunnett's multiple comparison tests, and significance was depicted by asterisks (*, $p < 0.05$; **, $p<0.01$).

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contribution

W.W. and L.S. conceived the studies, J.Y. conducted the majority of the experiments, and J.X. contributed to image taking by confocal microscope and X.W. contributed to cell culture. The manuscript was written by J.Y. and revised by L.S.

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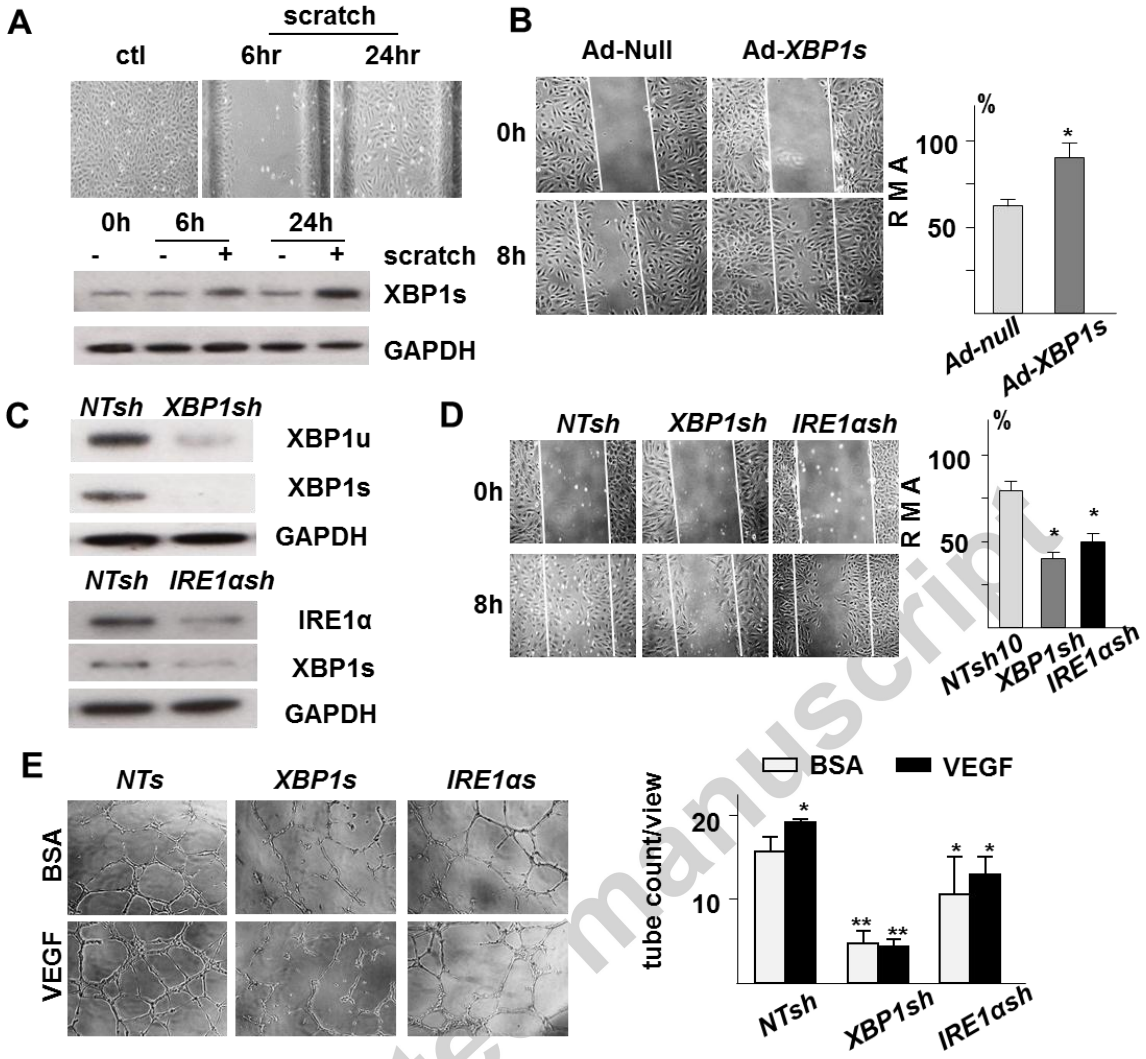


Figure 1. XBP1 splicing contributed to EC migration. (A) Wound induced XBP1 splicing. Wound was introduced into a confluent HUVEC monolayer by scratching, followed by observation of cell migration and western blot analysis of the spliced XBP1 (XBP1s) protein at 6h and 24h post-scratching. GAPDH was included as loading control. Scale bar: 100μm. **(B)** Over-expression of XBP1s enhanced EC wound healing. Confluent HUVECs were infected with Ad-null or Ad-*XBP1s* virus at 10 MOI for 24h, followed by scratching and observation of wound healing at time indicated. **(C-E)** Knockdown of *XBP1* or *IRE1α*attenuated wound healing and tube formation.XBP1 splicing could be down-regulated by *XBP1* shRNA (*XBP1sh*) or *IRE1α* shRNA (*IRE1α*) lentiviruses as revealed by Western blot **(C)**. The lentivirus-infected HUVECs (for 72h) were subjected to wound healing assays **(D)** or tube formation on Matrigel assays in the absence or presence of 10ng/ml VEGF **(E)**. Non-target shRNA (*NTsh*) lentivirus and 1% BSA were included as control. The relative

migrated area (RMA) was defined as the percentage of the area occupied by the migrated cells to that of the empty area created by the scratching. The data presented were representative images or average of three independent experiments. Scale bar: 100μm. *:p<0.05; **:p<0.01.

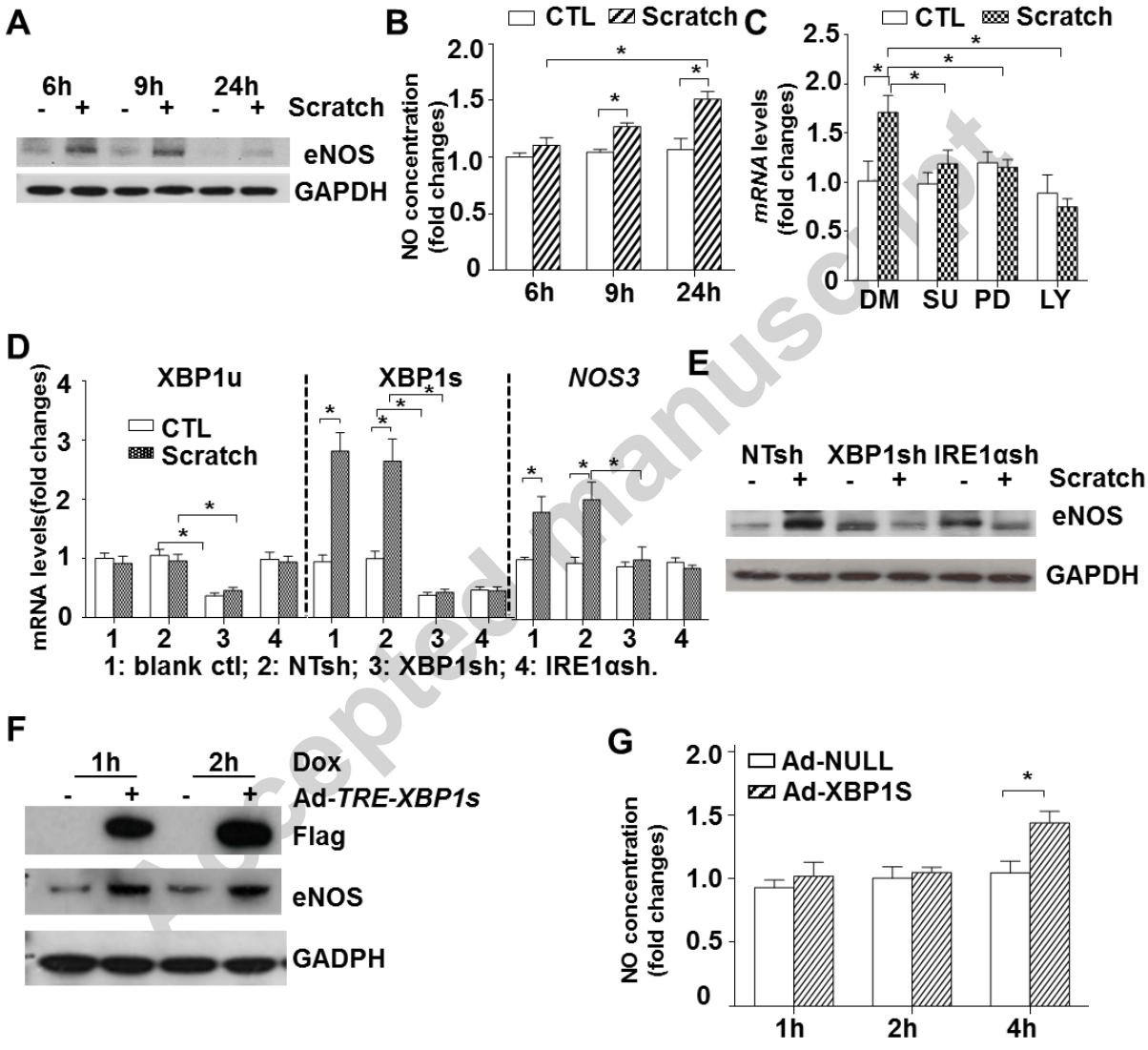


Figure 2. Wound in EC monolayer up-regulated eNOS expression in an XBP1 splicing-dependent manner. (A-B) Wound increased eNOS protein level (A) and NO production (B). Wound was introduced into a confluent HUVEC monolayer via scratching, followed by Western blot analysis of eNOS protein level (A) and NO production assay (B) at time

indicated post-scratching. **(C)** Inhibition of the VEGF signalling pathway abolished wound-induced *NOS3* expression. The confluent HUVEC monolayer was pre-treated with inhibitors for 1h, then subjected to scratching and incubation in the presence of inhibitors for 6hr, followed by quantitative RT-PCR analysis of *NOS3* mRNAs. DMSO (DM) was included as vehicle control. SU: SU1498, PD: PD98059, LY: LY294002. **(D)** Knockdown of *XBP1* or *IRE1α* abolished wound-induced *NOS3* upregulation. HUVECs were infected with non-target (*NTsh*), *XBP1* (*XBP1sh*) or *IRE1α*(*IRE1αsh*) shRNA lentivirus for 72h, followed by scratching and quantitative RT-PCR analysis of *XBP1u*, *XBP1s* and *NOS3* mRNA levels at 6h post-scratching. **(F-G)** Overexpression of XBP1s increased eNOS protein level and NO production. HUVECs were infected with Ad-*TRE-XBP1s* (10 MOI) together with Ad-Teton (2 MOI) for 24h, and then treated with 1ug/ml doxycycline (Dox) for time indicated, followed by Western blot analysis (F) and NO production assay (G). Ad-null was included as control. Anti-FLAG was included to indicate XBP1s expression. GAPDH was included as loading control. The data presented were representative images or average of three independent experiments. *: $p<0.05$.

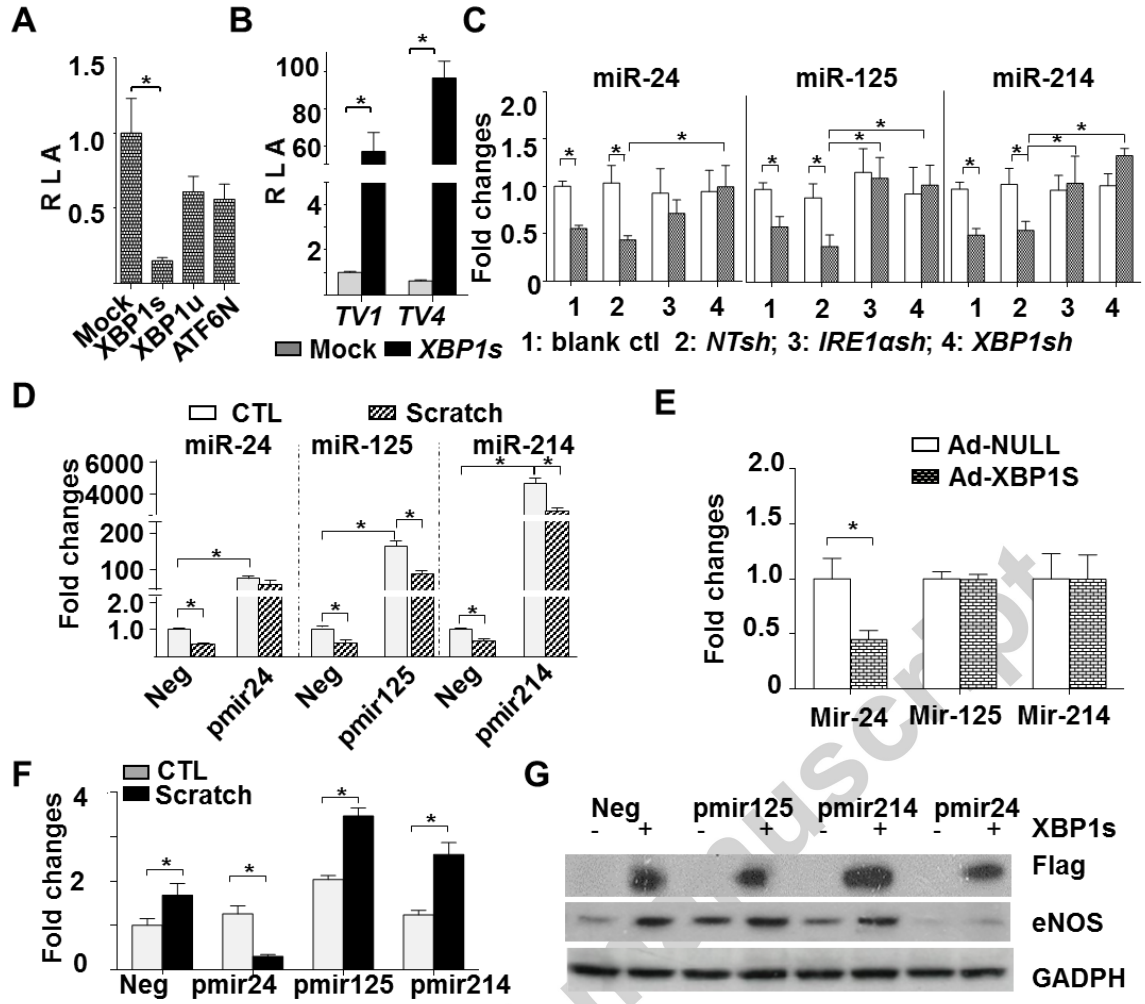


Figure 3. XBP1s increased *NOS3* mRNA stability via miR-24. (A) XBP1s suppressed pGL3-*NOS3-Luc* reporter expression. The relative luciferase activity (RLA) was defined as the ratio of firefly luciferase to renilla luciferase activity with that of mock group set as 1.0. **(B)** XBP1s increased pSiCHECK2-*TV1-3'UTR* and pSI-CHECK2-*TV4-3'UTR* reporter expression. The relative luciferase activity (RLA) was defined as the ratio of renilla luciferase to firefly luciferase activity with that of mock group set as 1.0. **(C)** Scratching down-regulated miR-24, miR-125 and miR-214 in an XBP1 splicing dependent manner. Quantitative RT-PCR analysis of miR-24, miR-125, miR-214 levels was performed at 6h post scratching. **(D)** Scratching increased miR-125 and miR-214 degradation. PremiRs (pmir) were introduced into HUVECs and subjected to scratching, followed by quantitative analysis of the miRNAs levels. **(E)** Over-expression of XBP1s down-regulated miR-24. HUVECs were infected with Ad-XBP1s (10 MOI) for 24h, followed by quantitative RT-PCR analysis. **(F)** Exogenous premiR-24 (pmir24) abolished scratch-induced *NOS3* mRNA upregulation. **(G)** Exogenous miR-24 abolished XBP1s-induced eNOS expression. HUVECs were

transfected with 10pmol/well premiR24, premiR125 and premiR214 respectively. 48h later, the transfected cells were infected with Ad-null or Ad-TRE-XBP1s/Ad-Teton virus at (10/2) MOI for 24h, and then treated with 1ug/ml doxycycline (Dox) for 1h, followed by Western blot analysis of eNOS and FLAG-tagged XBP1s expression. The data presented were representative images or average of three independent experiments. *: $p<0.05$.

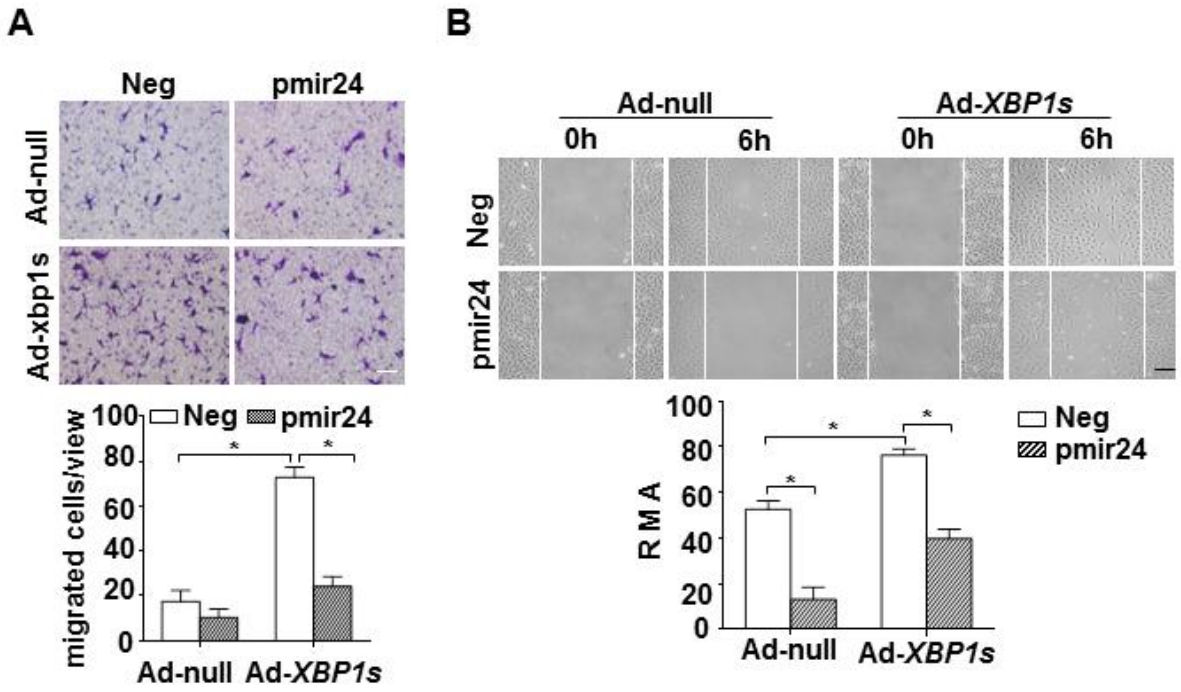


Figure 4. miR-24 was involved in XBP1s-induced ECs migration. HUVECs were transfected with premir24 for 6h, then infected with Ad-XBP1s (10 MOI) for 24h, followed by transwell migration assays (A) and wound healing assays (B). The relative migrated area (RMA) was defined as the percentage of the area occupied by the migrated cells to that of the empty area created by the scratching. Scale bar: 100μm. The data presented were representative images or average of three independent experiments. *: $p<0.05$.

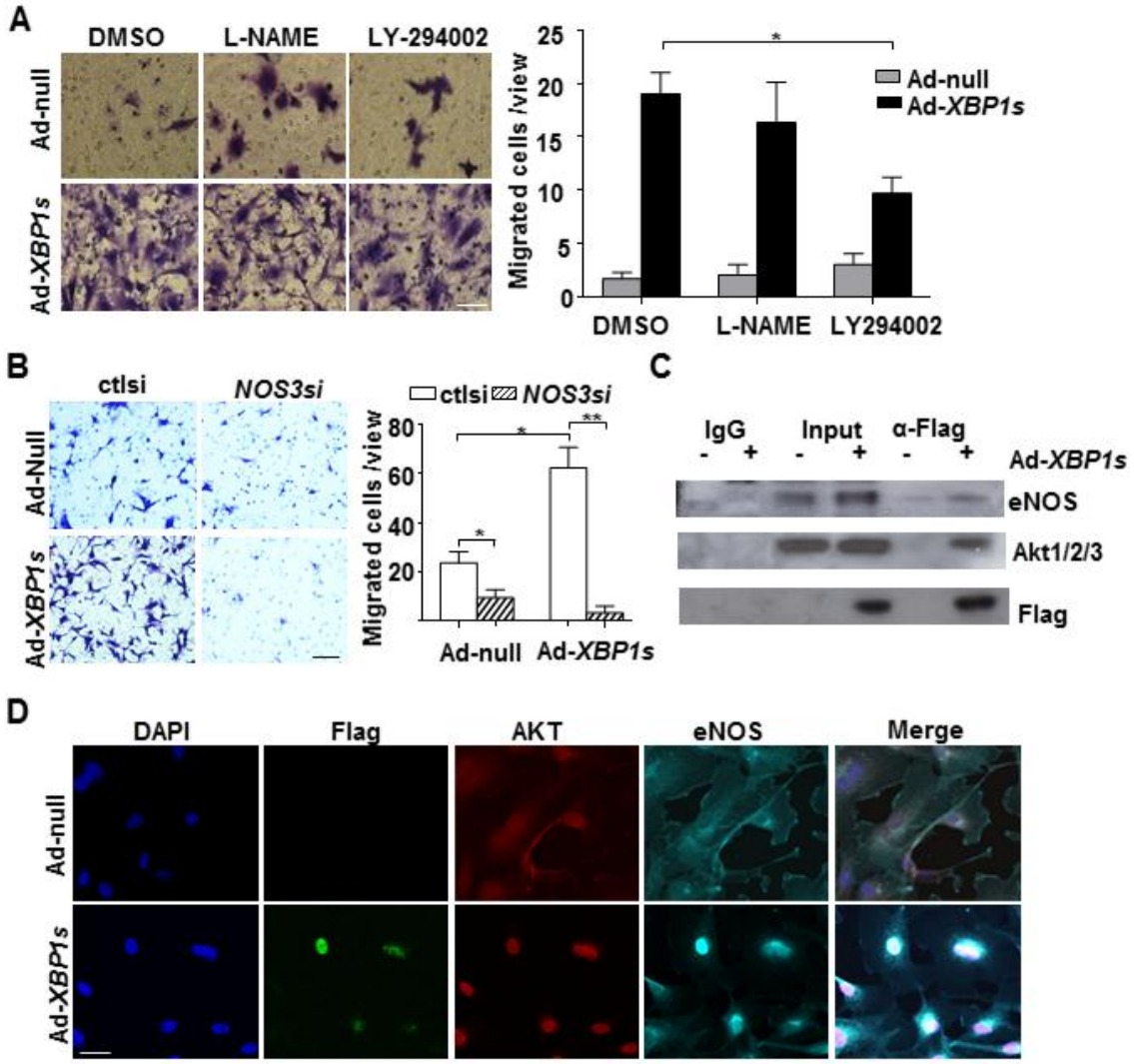


Figure 5. eNOS was involved in EC migration via forming a complex with AKT and XBP1s. (A) LY294002 but not L-NAME abolished XBP1s-induced EC migration. HUVECs were infected with Ad-null or Ad-XBP1s virus at 10 MOI for 24h, then pretreat with LY294002 (5μmol/L) and L-NAME (100μmol/L) for 2h, followed by trans-well migration assay in the presence of inhibitors for 6h. Migrated cells were calculated using 20x lenses view. DMSO was included as vehicle control. Scale bar: 25μm. **(B)** Knockdown of *NOS3*abolished XBP1s-inducedECs migration. HUVECs were transfected with NOS3 siRNAs for 48h, then infected with Ad-XBP1s for 24hr, followed by trans-well migration assay. Migrated cells were calculated using 10x lenses view. Control siRNA (ctlsi) and Ad-null were included as control. Scale bar: 50μm.**(C)** XBP1s, eNOS and Akt formed a complex. anti-Flag antibody was used to pulldown and detect XBP1s. **(D)** XBP1s, eNOS and Akt co-localised in the nucleus. Triple immunofluorescent staining was performed in Ad-null and

Ad-XBP1s infected HUVECs with anti-FLAG (green, for XBP1s), Akt (red), eNOS (purple). The nucleus was counter-stained with DAPI (blue). Scale bar: 10μm. The data presented were representative images or average of three independent experiments. *: $p<0.05$.

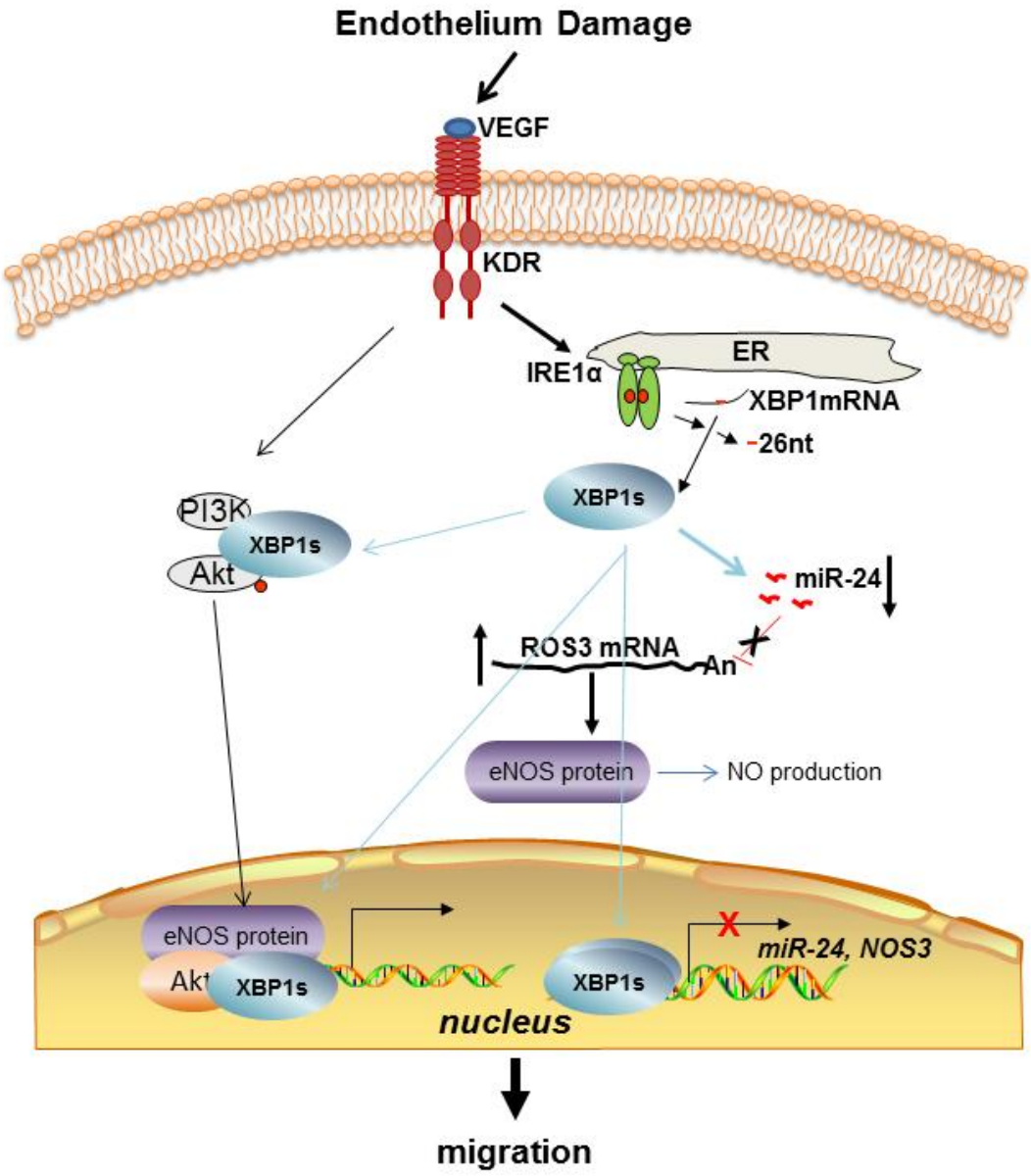
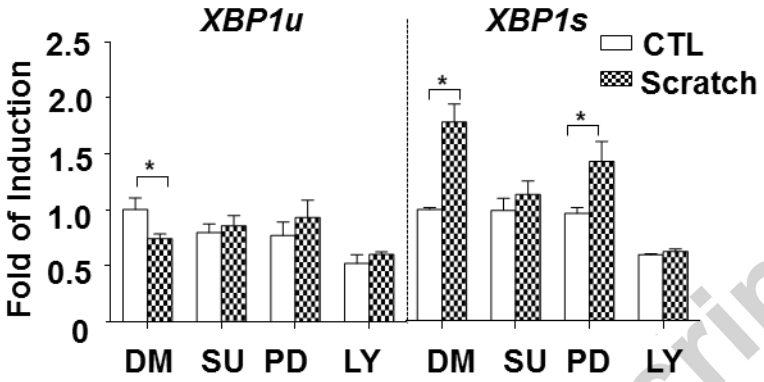
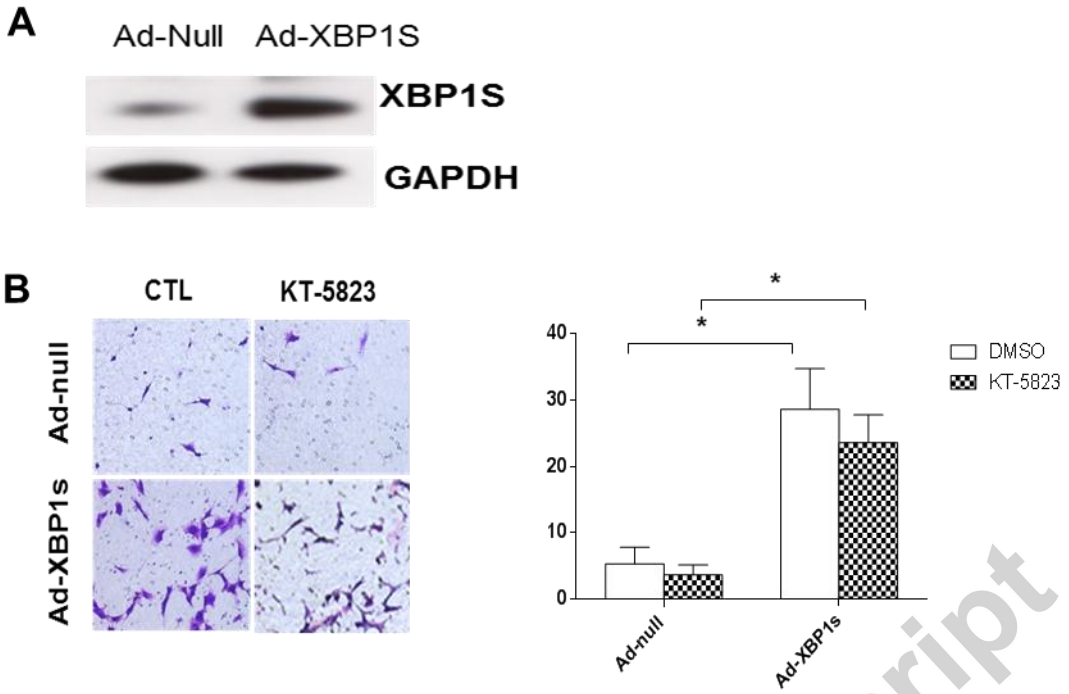


Figure 6. The interaction between XBP1 and eNOS contributes endothelium damage-induced EC migration. Endotheliumdamage activates VEGF receptor KDR in the adjacent ECs in a VEGF dependent or independent manner, which in turn activates IRE1α-mediated XBP1 mRNA unconventional splicing, removing a 26 nucleotide (nt) and causing the open reading frame to shift to produce a transcriptional active isoform, the XBP1s. XBP1s suppresses miR-24 transcription on one hand, leading to NOS3 mRNA stabilisation, eNOS protein and NO production increase. The elevated NOS3 mRNA may drive XBP1s

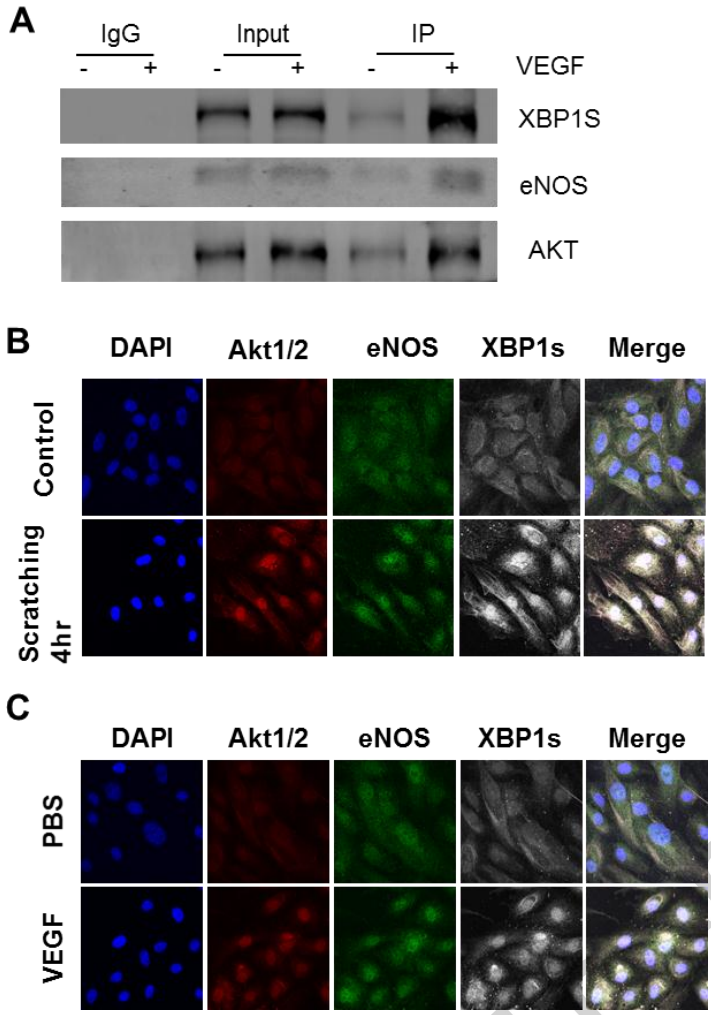
suppressing NOS3 transcription in a feed-back way. On the other hand, XBP1s may be involved in Akt activation and facilitate the nuclear relocation of Akt and eNOS protein to form an Akt/XBP1/eNOS complex in the nucleus, which may be involved in the transcription of genes that are involved in cell migration regulation. The overall effect is to increase the adjacent EC migration to repair the damaged endothelium.



Supplement 1.Wound in EC monolayer induced XBP1 splicing in a VEGF signalling pathway dependent manner.VEGF signalling pathway inhibitors abolished the scratching-induced XBP1 splicing. The confluent HUVEC monolayer was treated with inhibitors for 1h, then subjected to scratching and incubation in the presence of inhibitors for 6hr, followed by quantitative RT-PCR analysis of the unspliced (*XBP1u*) and spliced (*XBP1s*) *XBP1* mRNAs. DMSO (DM) was included as vehicle control. SU: SU5416 (5μmol/L), PD: PD98059 (5μmol/L), LY: LY294002 (5μmol/L). The data presented were representative images or average of three independent experiments. *: $p<0.05$.



Supplement 2. XBP1 splicing induced endothelial cell migration is PKG independent.(A) HUVECs were infected with Ad-XBP1s (10 MOI) for 24h, followed by Western blot analysis. **(B)** Infected HUVECs then pretreat with KT-5823(5μmol/L for 2h, followed by trans-well migration assay in the presence of inhibitors for 6h. Migrated cells were calculated using 20x lenses view. DMSO was included as vehicle control. Scale bar: 25μm. The data presented were representative images or average of three independent experiments. *: $p<0.05$.



Supplement 3：eNOS was involved in EC migration via forming a complex with AKT and XBP1s.(A)XBP1s, eNOS and Akt formed a complex with the treatment of VEGF. Anti-XBP1s antibody was used to pulldown. (B, C) XBP1s, eNOS and Akt co-localized in the nucleus. Triple immunofluorescent staining was performed in scratched (B) and VEGF(C) treated HUVECs with anti-Akt1/2 (red); anti-NOS3 (green), anti-XBP1s (gray). The nucleus was counter-stained with DAPI (blue). Scale bar: 10μm. The data presented were representative images or average of three independent experiments. *: $p<0.05$.

Abbreviations

X-box binding protein 1 (XBP1)

vascular endothelial growth factor (VEGF)

endothelial cell (EC)

XBP1 (XBP1s)

nitric oxide synthase 3 (*NOS3*)

adenovirus -XBP1s （Ad-XBP1s）

Protein kinase B (Akt)

nitric oxide synthase 3 (NOS3)

nitric oxide (NO)

basic-region leucine zipper (bZIP)

inositol requiring enzyme 1 alpha (IRE1α)

endoplasmic reticulum (ER)

unsplicedXBP1 (XBP1u)

human umbilical vein EC (HUVEC)

NOS3 promoter reporter expression (pGL3-*NOS3*-Luc reporter)

mature activating transcription factor 6 (ATF6N)

NG-nitro-L-arginine methyl ester (L-NAME)

the kinase insert domain receptor (KDR)

doxycycline (Dox)\

Dimethyl sulfoxide (DMSO, DM)

Highlights

- Wound in EC monolayer induced XBP1 splicing, which was involved in EC migration.
- XBP1s increased NOS3 mRNA stability via down-regulating microRNA miR-24.
- ENOS was involved in EC migration via forming a complex with AKT and XBP1s.